PATENT APPLICATION
DOCKET NO. 27866/32663

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	)	For: Methods for Using Agents that Bind to VCAM-1 (Amended Title)
Boris Masinovsky et al.	)	
Serial No: 08/448,649	)	Group Art Unit: 1815
Filed: May 24, 1995	)	Examiner: P. Gambel, Ph.D.

SECOND DECLARATION OF BEVERLY J. TOROK-STORB, Ph.D., UNDER 37 C.F.R. §1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

I, Beverly J. Torok-Storb, Ph.D., hereby declare as follows that:

1. I received a B.S. in biology and secondary education in 1970 and a master's degree in Secondary Education in 1971 from Edinboro State College, Edinboro, Pennsylvania. I received a Ph.D. in radiation biology and human genetics in 1975 from the University of Pittsburgh Graduate School of Public Health, Pittsburgh, Pennsylvania. From 1975 to 1976 I was a Senior Fellow at the University of Washington Department of Pathology, Seattle, Washington. At the University of Washington School of Medicine, I was a Senior Fellow at the Department of Hematology from 1976 to 1979, a Research Assistant Professor of Medicine from 1979 to 1985, a Research Associate Professor of Medicine from 1985 to 1991, and a Research Professor since 1991. At the Fred Hutchinson Cancer Research Center, Seattle, Washington, I was an Associate in Medical Oncology from 1978 to 1980, an Assistant Member from 1980 to 1984, an Associate Member from 1984 to 1991, and a Member since 1991.

I am an author or co-author of over 85 scientific publications. My honors and activities include: a National Research Service Fellowship Award from NIAMDD in 1977; a Special Fellowship from the Leukemia Society of America in 1979; a Young Investigator Award from NHLBI in 1979; Chairman of Clinical Sciences Study Section 4 (CLN4), National Institutes of Health (NIH), from 1985 to 1988; Member of the NIH Reviewer's Reserve from 1988 to 1992; Visiting Professor at the Canadian Royal Academy of Physicians and Surgeons in 1989; Visiting Professor at the University of Ulm, Ulm, Germany in 1990; Chairman of the Scientific Committee ISEH meeting in Seattle in 1990; Member of the Science Advisory Council, Pacific Science Center in 1990.

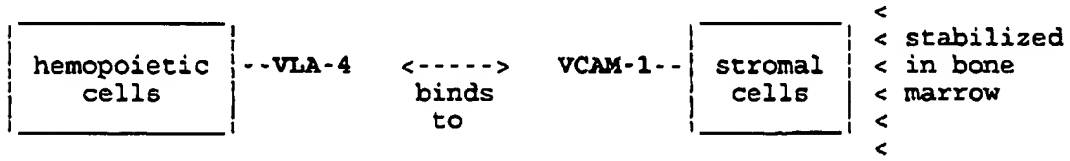
2. I have reviewed the above-identified patent application U.S. Serial No. 08/448,649, including the currently pending claims, and pages 2-3 of the Office Action dated March 20, 1997. As a result of my scientific training and experience, I am knowledgeable about bone marrow transplantation and about the interactions between bone marrow cells and bone marrow stromal cells. I am therefore qualified to discuss what one of ordinary skill in the art of bone marrow transplantation would understand from the statements made in the application as of its effective filing date, which I have been advised is August 2, 1990.

3. I make the following statements to respond to the Examiner's rejection of the currently pending claims on the basis that they are not described in the application as originally filed. Specifically, I respond to the Examiner's statements at pages 2-3 of the Office Action that:

> Applicant's amendment, filed 12/12/96 (Paper No. 23), has directed support for the amended claims to (1) page 4, lines 11-16; however said passage refers to blocking lymphocyte binding to activated bone marrow stromal cells and not hemopoietic precursors; (2) page 14, lines 28-32 which similarly refers to blocking lymphocyte adhesion and not hemopoietic precursors; and (3) page 17, lines 24-33 which refers to the expression of VLA-4 on human CD34+ bone marrow cells and infer that VCAM-1-VLA-4 interactions occur between hemopoietic cells and stromal elements and go on further to disclose the use of VCAM-1-specific antibodies to prevent GVHD.
>
> There does not appear to be support for interfering with hemopoietic cell-stromal cell interactions with VCAM-1-specific antibodies nor is there support how the skilled artisan would use such procedures. The inhibition of adhesion mediated by VCAM-1-specific antibodies as disclosed in the specification as filed is directed towards inhibiting lymphocyte adhesion such as useful in inhibiting GVHD and not towards inhibiting hemopoietic stem and/or progenitor cell adhesion. [Emphasis in original.]

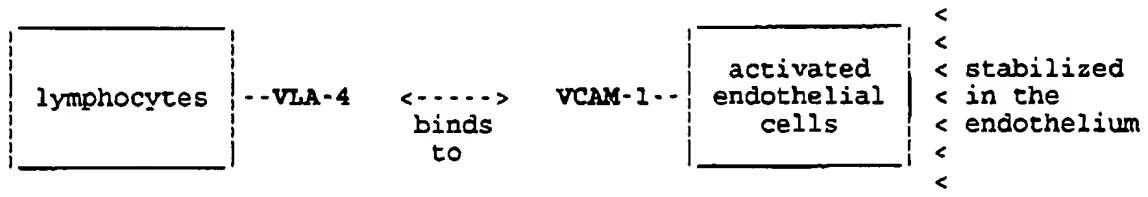
4. I agree with the Examiner's statement that the application, particularly at page 17, lines 24-33, "refers to the expression of VLA-4 on human CD34+ bone marrow cells and infer[s] that VCAM-1-VLA-4 interactions occur between hemopoietic cells and stromal elements." This interaction between hemopoietic cells (which express VLA-4) and bone marrow stromal cells (which express VCAM-1) is illustrated below (Fig. 1).

FIG. 1



5. In addition to disclosing Applicants' discovery of the role of VCAM-1 in the adhesive interaction between hemopoietic precursor cells and bone marrow stromal cells, the application also discloses that VCAM-1 mediates other adhesive interactions, such as those between lymphocytes (which express VLA-4) and activated endothelial cells (which express VCAM-1). This interaction is illustrated below (Fig. 2).

FIG. 2



The application (*e.g.*, at pages 4 and 15) also shows that anti-VCAM-1 antibodies such as 6G10 are able to block VCAM-1-mediated adhesion between lymphocytes and activated endothelial cells by up to 80%.

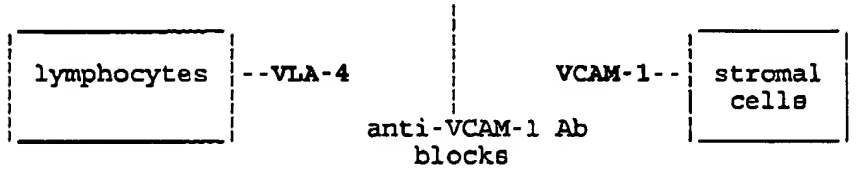
6. I strongly disagree with the Examiner's statement that the disclosed use of VCAM-1-specific antibodies to inhibit VCAM-1-mediated adhesion "is directed towards inhibiting lymphocyte adhesion . . . and not towards inhibiting hemopoietic stem and/or progenitor cell adhesion." There is no indication in the application that the use of VCAM-1-specific antibodies to interfere with, or block, intercellular adhesive interactions is limited to only some of the disclosed VCAM-1-mediated interactions and not others. On the contrary, one of ordinary skill in the art would understand from reading the application that anti-VCAM-1 antibodies are useful for blocking any VCAM-1-mediated adhesion, regardless of the type of cells involved.

7. For example, the application states at page 4, lines 11-16 that:

> The binding partners are preferably also characterized by the ability to block lymphocyte binding to cytokine-activated endothelial cells, and most preferably by binding to human VCAM-1 and to IL4- or TNFα-activated bone marrow stromal cells. A representative embodiment of this most preferred binding partner is mAb 6G10 . . .

The Examiner has characterized this passage as referring to "blocking lymphocyte binding to activated bone marrow stromal cells and not hemopoietic precursors." This statement is illustrated below.

FIG. 3



I disagree with the Examiner. One of ordinary skill in the art as of August 2, 1990 would have clearly understood upon reading the application, particularly the quoted portion at page 4, lines 11-16, that the Applicants taught use of anti-VCAM-1 antibody to inhibit any VCAM-1-mediated adhesive interaction, including the disclosed adhesive interaction between stromal cells and hemopoietic precursor cells (Fig. 1) and the disclosed adhesive interaction between lymphocytes and activated endothelial cells (Fig. 2). One of ordinary skill in the art would have extrapolated results associated with blocking one VLA-4-VCAM-1 interaction, *e.g.*, that shown in Fig. 2, to another VLA-4-VCAM-1 interaction, *e.g.*, that shown in Fig. 1 or Fig. 3.

8. I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the above-identified application or any patent issuing thereon.

Date: 8/19/97

Beverly J. Torok-Storb, Ph.D.

Adhesion Receptors on Bone Marrow Stromal Cells: In Vivo Expression of Vascular Cell Adhesion Molecule-1 by Reticular Cells and Sinusoidal Endothelium in Normal and γ-Irradiated Mice

By Karen Jacobsen, Joel Kravitz, Paul W. Kincade, and Dennis G. Osmond

Cell adhesion molecules (CAMs) play a key role in interactions between stromal and hematopoietic cells in bone marrow (BM) and in cell traffic through vascular endothelium. To examine the identity of CAMs involved in these processes in mouse BM, we have investigated the in vivo expression of vascular cell adhesion molecule-1 (VCAM-1) and its counterreceptor, very late antigen-4 (VLA-4). Radioiodinated monoclonal antibodies (MoAbs) detecting VLA-4 and VCAM-1 were injected intravenously. Antibody binding was detected in BM by light and electron microscope radioautography. VCAM-1 labeling was restricted to stromal reticular cells and endothelial cells lining BM sinusoids. VCAM-1$^+$ reticular cells formed patchy concentrations, especially in subosteal regions, associated with lymphoid, granulocytic, and erythroid cells. After γ-irradiation to deplete hematopoietic cells, reticular cells and endothelial cells all showed VCAM-1 labeling in apparently increased intensity. VLA-4 labeling was shown by undifferentiated blast cells and lymphohematopoietic cells both in BM cell suspensions and in vivo, especially at reticular cell contact points. The results demonstrate that VCAM-1 is expressed in vivo by certain BM reticular cells, suggesting that the molecule mediates adhesion to multiple lineages of lymphohematopoietic cells. The finding that VCAM-1 is also expressed constitutively by BM sinusoidal endothelium, unlike its inductive expression by endothelia elsewhere, suggests that VCAM-1 and VLA-4 may be involved in regulating the normal cell traffic between BM and the blood stream.

STROMAL CELLS of bone marrow (BM) provide a microenvironment that supports lymphohematopoiesis and regulates cell traffic. Some stromal cells provide growth signals to precursor cells of B lymphoid and myeloid lineages.[1-3] This interaction requires adhesion and the release of factors at the cell interfaces.[3-6] Other cells, notably endothelial cells of venous sinusoids, control the passage of cells to and from the blood stream.

The in vivo organization of BM stroma is complex. Stromal reticular cells permeate the extravascular space.[7-9] Lymphohematopoietic cells differentiating near the surrounding bone tend to move centrally as they develop, adhering to the reticular cell network.[10-13] At appropriate stages of maturation, the developing cells detach from the reticular cells and traverse the sinusoidal endothelium. Newly formed B lymphocytes, unlike other emigrants, are retained in the sinusoid lumen before circulating to the peripheral lymphoid tissues.[10-14] In addition, the sinusoidal endothelium permits the selective entry from the blood of plasmablasts, recirculating lymphocytes, hematopoietic stem cells, and some neoplastic cells, notably multiple myeloma cells.[15-18] Receptor molecules mediating the adhesive properties of stromal cells and endothelial cells in vivo are, thus, of central importance in lymphohematopoiesis and the interchange of cells between BM and blood.

Vascular cell adhesion molecule-1 (VCAM-1) is a member of the immunoglobulin family of cell adhesion molecules (CAMs). It is constitutively expressed by dendritic cells and follicular dendritic cells (FDC) in peripheral lymphoid tissues, and it can be induced on either cultured or cytokine-activated endothelial cells.[19-25] Correlating with this pattern of expression, VCAM-1 has been implicated in mediating certain cell interactions in normal lymphoid tissues and germinal centers and the migration into inflammatory sites and neoplasms of blood cells that express the counterreceptor very late antigen-4 (VLA-4), a β1 integrin. VCAM-1 is also expressed by cultured BM stromal cells.[26,27] Anti–VCAM-1 antibodies cause lymphohematopoietic precursors and leukemia/lymphoma cell lines to detach from cultured BM stromal cells, inhibiting B lymphopoiesis and myelopoiesis.[26,27] Anti–VLA-4 antibodies have similar effects.[28] The finding that VCAM-1/VLA-4 interactions are functionally important in BM cultures has raised the possibility of a similar role in vivo. There appear to have been no comparable studies of CAMs on the specialized endothelium of BM sinusoids.

The present study was aimed at examining the expression of VCAM-1 and VLA-4 by BM cells under physiologic circumstances in vivo. We have administered radiolabeled anti–VCAM-1 and anti–VLA-4 monoclonal antibodies (MoAbs) intravenously to mice and have detected MoAb-binding in BM by electron microscope radioautography. This technique permits the localization of cell surface determinants with high resolution.[10-14] MoAb M/K-1, prepared against BM stromal cells, recognizes VCAM-1 and produces a reticular staining pattern on frozen sections of BM.[26] MoAb PS.2, prepared against lymphomyeloid cells, recognizes VLA-4.[28] Using these MoAbs in normal mice and in γ-irradiated mice to reveal details of the radioresistant BM stroma, we now show that VCAM-1 is constitutively expressed both by BM reticular cells, associated with a variety of VLA-4$^+$ hematopoietic lineages, and by the entire endothelium of BM sinusoids.

MATERIALS AND METHODS

Animals. Male C3H/HeJ mice, 3 to 5 weeks old, were purchased from Jackson Laboratories (Bar Harbor, ME) and kept in microisola-

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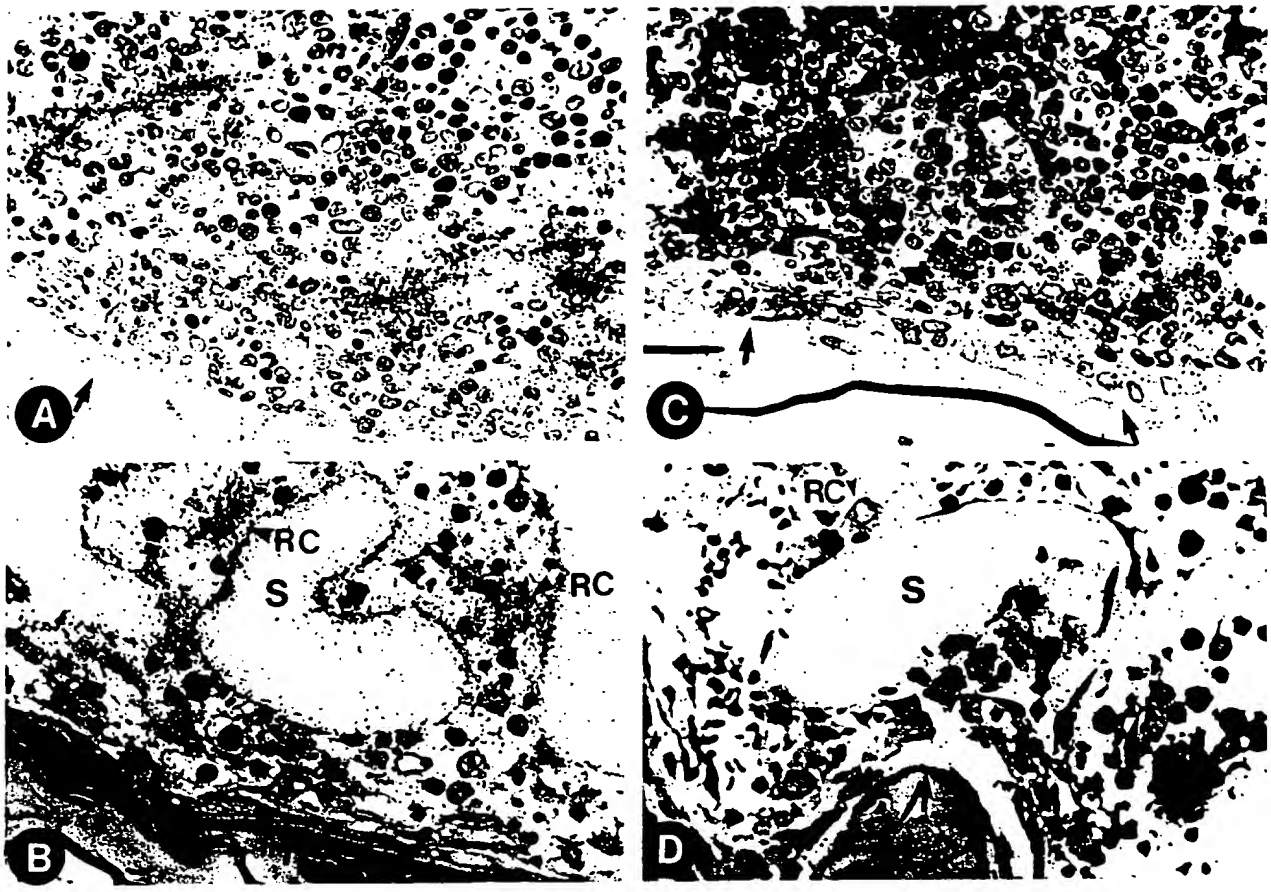

Fig 1. Radioautographic sections of femoral BM and adjacent bone (arrows) after in vivo injection of ^{125}I-MAbs M/K-2 (A, B) and PS/2 (C, D) in normal (A, C) and γ-irradiated (B, D) mice. (A) Linear concentrations and patches of MAb M/K-2 labeling. (B) Heavy M/K-2 labeling of reticular cells (RC) and endothelium of dilated sinusoids (S). (C) Low intensity labeling of many hematopoietic cells by MAb PS/2. (D) A cluster of MoAb PS/2-labeled cells having vacuolated cytoplasm and eccentric nuclei within a sinusoid. The few radioresistant extravascular cells show little or no labeling (radioautographic exposure, 14 days; original magnification [OM], × 670).

tors. After irradiation, mice were fed autoclaved food and acidified water.

Irradiation. Mice in Plexiglas holding boxes were given a single dose of 9.5 Gy whole-body γ-irradiation (Cs^{137}, 1.21 Gy/min) using a Gammacell irradiator (Radiation Safety Canada, Ottawa, Canada). This dose ablates all but a small proportion of hematopoietic cells in BM while leaving radioresistant stromal cells intact, thus allowing normally obscure stromal cell processes to be identified clearly by microscopy.

Antibodies and radioiodination. Affinity-purified MoAbs M/K-2 and PS/2 were prepared as described.[26,28] Each antibody (150 μg) was radioiodinated by a modified chloramine-T method, as described,[12] giving a final concentration of 5 to 10 μg/100 μL (specific activity: MoAb M/K-2, 4.5 × 10^6 cpm/mg; MoAb PS/2, 2.7 × 10^6 cpm/mg).

In vivo labeling by MoAbs M/K-2 and PS/2. Three control and three γ-irradiated mice were anesthetized with 1.6% chloral hydrate in 0.9% sterile saline (intraperitoneally: 0.03 mL/g body weight). The external jugular vein was exposed, and 100 μL of ^{125}I-labeled MoAb was injected intravenously, as described.[12] After 3 minutes, unbound MoAb and blood were washed out by whole-body arterial perfusion. The left ventricle was cannulated and a syringe pump (model 355; Sage Instruments, White Plains, NY) was used to deliver cold Ringer's solution (3 mL/min; 10 minutes), which escaped from an incision in the right atrium. Tissues were then fixed in situ by perfusing cacodylate-buffered 2.5% glutaraldehyde (pH 7.2) for 10 minutes.

Tissue processing and radioautography. Perfusion-fixed femurs were immersed in buffered 2.5% glutaraldehyde fixative overnight, decalcified in EDTA (10% wt/vol) at 4°C for 5 days, postfixed in 1% potassium ferrocyanide-reduced osmium tetroxide, dehydrated in acetone, and embedded in Epon 812 (JBEM, St Laurent, Canada). Hardened Epon blocks were trimmed to expose a middiaphyseal face of femoral BM. For light microscopic (LM) preparations, sections (1-μm thick) were stained with iron alum and hematoxylin and dipped in Kodak K5D emulsion (Kodak, Toronto, Canada). LM radioautographs were developed after exposure for 8 and 21 days. For electron microscopy (EM), sections (70 to 84 nm thick) were placed on celloidin-coated glass slides, dipped in Ilford L4 emulsion (Ilford, Essex, UK), and exposed for 6, 10, and 14 weeks. After radioautographic development, the sections were placed on copper grids and stained with uranyl acetate and lead citrate, as described.[12] LM radioautographs were examined for the intensity and distribution of radioautographic grains using a Zeiss photomicroscope with bright field illumination (Zeiss Canada, Toronto, Canada). Using a transmission electron microscope (Phillips EM 400, Eindhoven, The Netherlands), at least three entire sections from each femur of three mice were examined at each of the three exposure times, representing a scrutiny

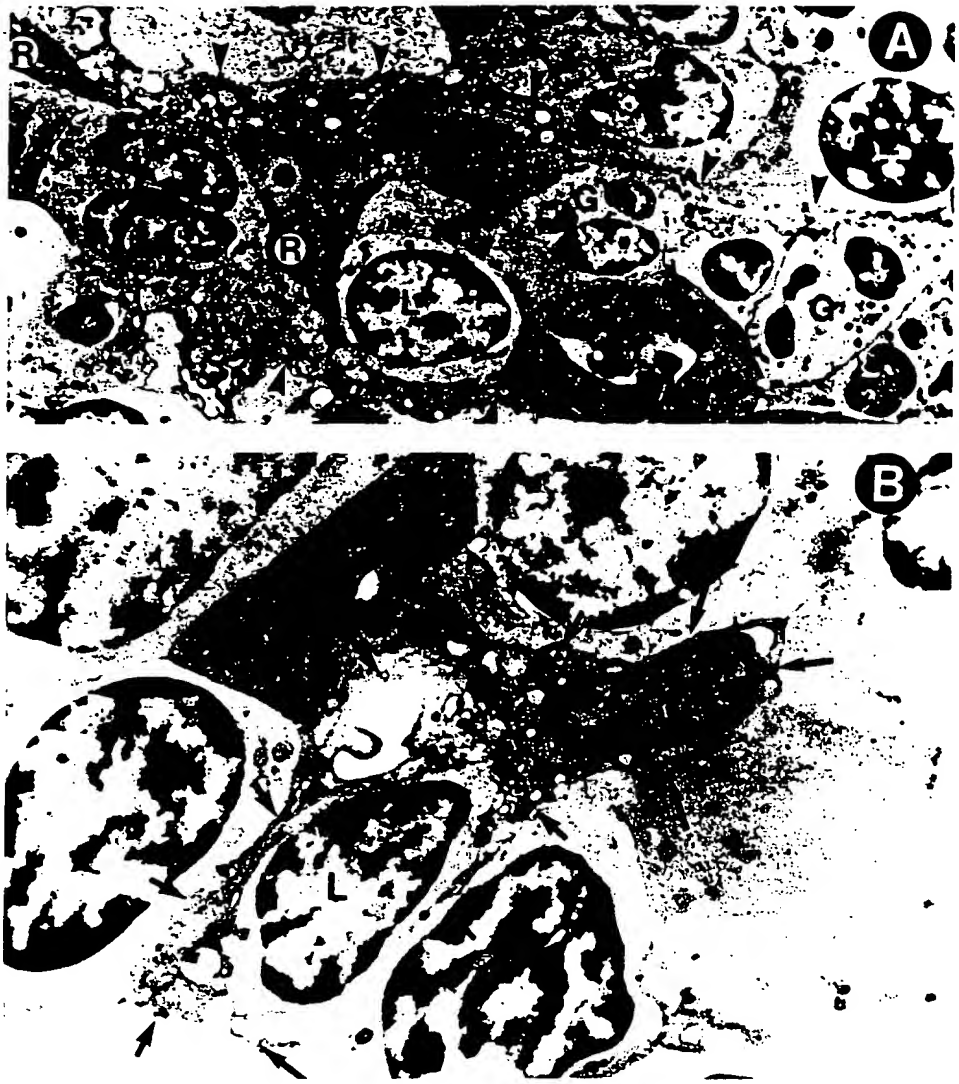

Fig 2. EM radioautographs of cells binding ^{125}I-MoAb M/K-2 in BM. (A) Stromal reticular cells with irregular nuclei (R) and electron-dense vacuolated cytoplasmic processes showing VCAM-1 labeling along their entire length (arrows). The processes are associated with various hematopoietic cells (lymphoid, L; erythroid, E; and granulocytic, G; OM, × 3,900). (B) A stromal reticular cell (RC) with VCAM-1–labeled processes (arrows) partially surrounds a lymphoid cell (L) (radioautographic exposure, 14 weeks; OM, × 8,600).

of more than 150,000 nucleated BM cells for each MoAb. The same number of BM sections, composed mostly of stromal cells, were examined from irradiated mice.

In vitro labeling of cell suspensions by MoAbs M/K-2 and PS/2. Two groups of three mice were killed by cervical dislocation. Femurs and spleens were removed and placed in cold Eagle's minimum essential medium containing 10% newborn calf serum (NCS/MEM). Spleens were teased through wire mesh and thoroughly resuspended in cold NCS/MEM. Femoral epiphyses were removed, and diaphyseal BM was flushed out with cold NCS/MEM. Cell suspensions were layered over NCS to allow particles of bone and connective tissue to sediment out and were washed by centrifugation through NCS (7 minutes, 200 *g*). The cells were resuspended in NCS/MEM and adjusted to a concentration of 4×10^7 mL, measured by an electronic particle counter (Coulter Electronics, Hialeah, FL). Cell suspensions (100 μL) were incubated with either ^{125}I-MoAb M/K-2 or ^{125}I-MoAb PS/2 (100 μl; 1:50 dilution) for 30 minutes on ice, washed through 6% bovine serum albumin (BSA) in phosphate-buffered saline (PBS, pH 7.2; 7 minutes 200 *g*), resuspended in 6% BSA/PBS, smeared onto gelatin-coated glass slides, and air-dried. The slides were dipped in Kodak NTB-2 emulsion (Kodak), exposed for 1, 8, and 21 days, developed, and stained with a modified MacNeal's tetrachrome. A total of 1,000 to 2,000 nucleated cells were examined throughout the length of each smear, and the radioautographic grains associated with each cell were counted. Cells were assigned to erythroid, granulocytic, and lymphoid lineages by established hematologic criteria.[29]

RESULTS

In vivo binding of MoAbs M/K-2 and PS/2 to BM cells. The in vivo administration of radiolabeled MoAb M/K-2, recognizing VCAM-1, and MoAb PS/2, recognizing VLA-4, produced contrasting patterns of LM radioautographic labeling in BM of young C3H/HeJ mice (Fig 1). Discrete areas of labeling by ^{125}I-MoAb M/K-2 were present throughout transverse sections of midshaft femoral BM (Fig 1A). Linear concentrations of labeling, apparently associated with venous sinusoids, were located in all areas, while patches of heavy extravascular labeling were also present, most prominently in peripheral areas near the surrounding bone. In contrast, ^{125}I-MoAb PS/2 administration produced a generalized low-intensity labeling of extravascular cells throughout the BM (Fig 1C).

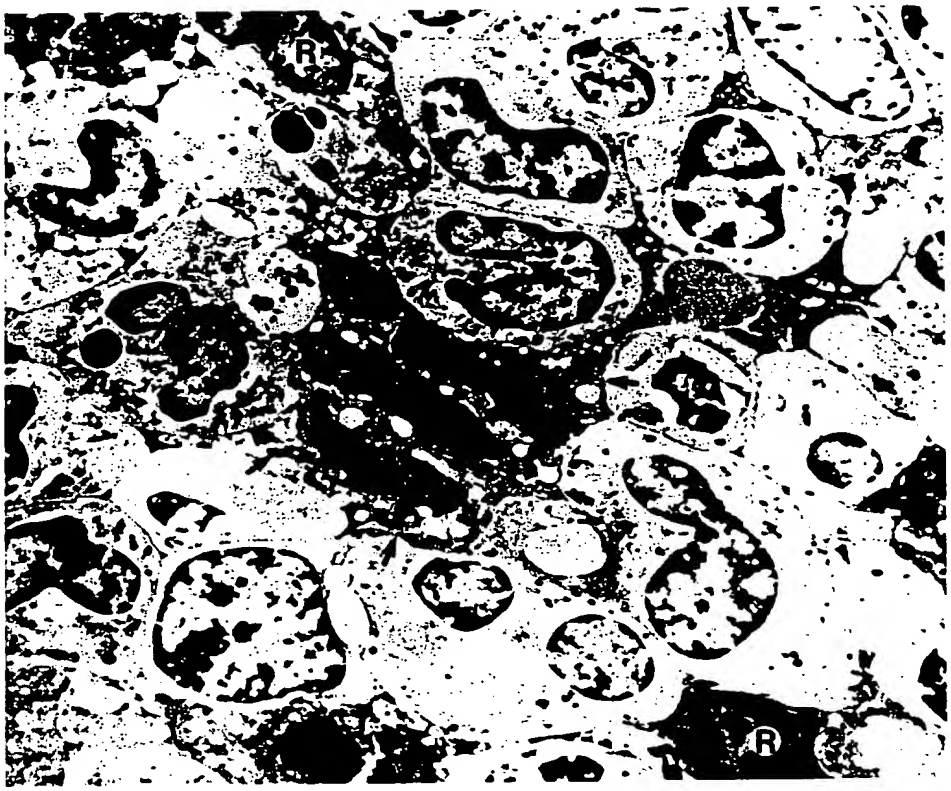

Fig 3. A cluster of VCAM-1$^+$ reticular cells (arrows) and two unlabeled reticular cells (R) in BM (radioautographic exposure, 14 weeks; OM, × 5,600).

To distinguish between labeling of BM stromal cells and hematopoietic cells, young C3H/HeJ mice were γ-irradiated (9.50 Gy) 2 days before administering radiolabeled MoAbs M/K-2 or PS/2. Nearly all hematopoietic cells had been killed by γ-irradiation and removed from BM, revealing the radioresistant stromal elements (Fig 1B and D). The extravascular compartment, reduced to narrow spaces between dilated venous sinusoids, contained stromal reticular cells and macrophages interconnected by branching cytoplasmic processes (Fig 1B and D). The few remaining hematopoietic cells included megakaryocytes, granulocytes, and cells of undifferentiated morphology. The BM of γ-irradiated mice showed intense MoAb M/K-2 binding by many reticular cells and their processes, as well as by sinusoidal endothelium throughout the BM (Fig 1B). The binding of MoAb PS/2, in contrast, was minimal. A few residual hematopoietic cells (three to five per BM section) and some macrophages containing numerous phagosomes showed MoAb PS/2 labeling, whereas reticular cells and sinusoidal endothelium were unlabeled. Some cells of uncertain identity within the sinusoidal lumen were labeled by MoAb PS/2 (Fig 1D).

VCAM-1 expression by stromal cells in BM. Details of ^{125}I-MoAb M/K-2 binding were revealed by EM radioautography after giving the MoAb to normal mice (Figs 2 through 5). Heavy VCAM-1 labeling was restricted to reticular cells characterized by an electron-dense cytoplasm containing clear vacuoles and heterogeneous granules and an elongated nucleus exhibiting many clumps of heterochromatin (Figs 2 through 4). The intensity of VCAM-1 expression, indicated by density of radioautographic labeling, was approximately uniform along the length of the reticular cell processes. Not all electron-dense stromal cells showed VCAM-1 labeling, however, and the numerous electron-lucent reticular cells were generally VCAM-1–negative.

Typically, each VCAM-1$^+$ reticular cell process was applied closely to a number of hematopoietic cells, which by morphologic criteria represented a variety of lineages (lymphoid, granulocytic, erythroid) and differentiation stages (Fig 2A). Many VCAM-1$^+$ reticular cells were located in peripheral areas of BM, occasionally forming interconnected networks, in some cases surrounding a cluster of hematopoietic cells (Fig 2A and B). Some VCAM-1$^+$ reticular cells in the subosteal region were in small groups (Fig 3), while others appeared either to form an adventitial cover around subendosteal capillaries, or partially to replace the endothelial lining of venous sinusoids (not shown).

Endothelial cells of BM sinusoids were prominently labeled by ^{125}I-MoAb M/K-2 (Fig 4). All such endothelium was labeled, from small subosteal sinusoids to the large central venous sinus. The intensity of VCAM-1 labeling was uniformly heavy over each endothelial cell. EM radioautographic grains were closely superimposed over the endothelium, but, because of the tenuous nature of the cells, the VCAM-1 expression generally could not be assigned to either the luminal or the abluminal surface. Where endothelial cells thickened, however, particularly near the nucleus, VCAM-1 expression was clearly evident on the luminal surface (Fig 4). Arteriolar endothelium also showed some light labeling by ^{125}I-MoAb M/K-2.

Hematopoietic cells remained unlabeled by ^{125}I-MoAb M/

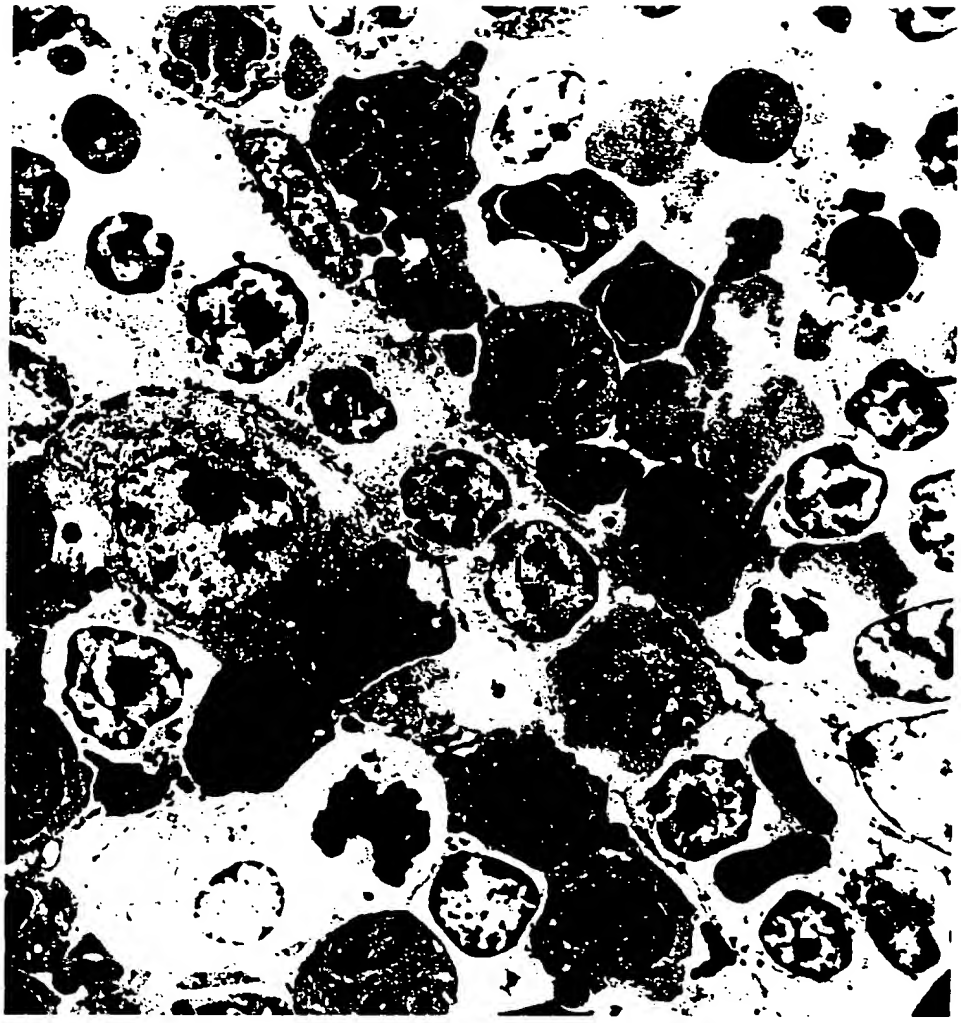

Fig 4. VCAM-1$^-$ endothelial cells (E) lining a BM sinusoid containing eight small lymphocytes (L) (radioautographic exposure, 14 weeks; OM, × 3,040).

K-2 except that peripherally-located megakaryocytes showed labeling around their circumference, often associated with processes of labeled reticular cells (not shown).

VCAM-1 expression by stromal cells in γ-irradiated BM. Reticular cell processes formed a network almost devoid of hematopoietic cells in BM of mice 2 days after γ-irradiation (9.50 Gy), as viewed by EM (Fig 5). The processes showed well-localized labeling by ^{125}I-MoAb M/K-2 (Fig 5). Compared with normal BM, the reticular cells were less heterogeneous in appearance, with none having a particularly electron-dense cytoplasm (Fig 5). Using a standard dose of ^{125}I-MoAb M/K-2 and identical radioautographic exposure times for both normal and γ-irradiated BM, the number of photographic grains overlying the reticular cells would be proportional to the radioactivity of bound MoAb M/K-2. By these criteria, the high labeling intensity displayed by reticular cells in irradiated BM (Fig 5) suggested an apparently increased expression of VCAM-1 compared with reticular cells in normal BM. Moreover, every reticular cell was labeled, in contrast to the selective labeling in normal mice.

Sinusoidal endothelium in γ-irradiated mice showed heavy VCAM-1 expression by EM radioautography (Fig 5). Some BM macrophages were lightly labeled by ^{125}I-MoAb M/K-2 (not shown) whereas the few residual hematopoietic cells were not appreciably labeled (Figs 1B and 5).

VLA-4 expression by hematopoietic cells in BM. To examine the range of hematopoietic cells binding MoAb PS/2, BM cell suspensions were exposed to ^{125}I-MoAb PS/2 in vitro and examined in radioautographic smears. One quarter (26%) of all nucleated cells in BM were labeled, many with high grain counts (greater than 25 grains). One half of the labeled cells were large cells of lymphoid morphology, a category of cells that includes early precursors of various lineages, particularly, precursor B cells.[30] MoAb PS/2 was bound by approximately one quarter of all small lymphocytes. These cells consist of IgM$^+$ B lymphocytes and small, postmitotic pre-B cells expressing cytoplasmic μ chains of IgM.[31] Substantial proportions of myeloid and erythroid cells were also labeled, especially at early stages of differentiation. Promyelocytes and basophilic erythroblasts were most intensely labeled. In contrast, exposure of BM cell suspensions to ^{125}I-MoAb M/K-2 produced no heavy labeling of any hematopoietic cells. Some labeling of near-threshold intensity on small lymphocytes, large lymphoid cells, and

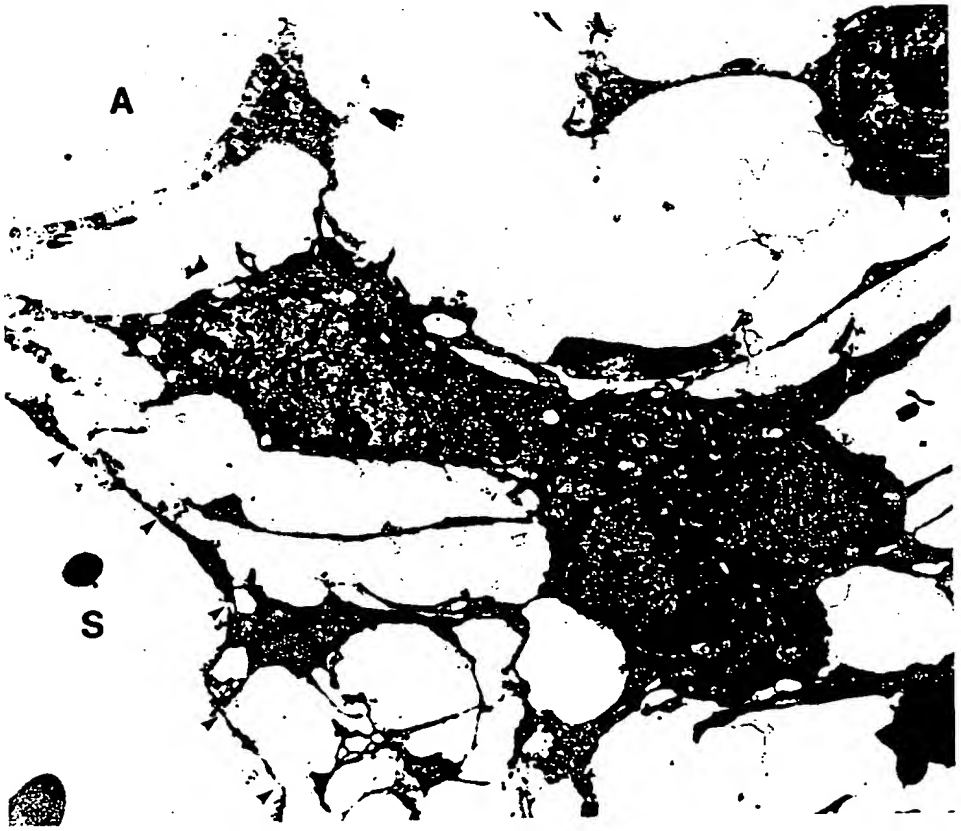

Fig 5. VCAM-1$^+$ reticular cells (RC) and endothelial cells (arrowheads) lining a sinusoid (S) in BM 2 days after whole-body γ-irradiation. A portion of an adipocyte (A) and an unidentified hematopoietic cell (H) are unlabeled (radioautographic exposure, 14 weeks; OM × 5,600).

myeloid cells in each case represented approximately 2% of nucleated BM cells, while the incidence of such labeled erythroid cells was 0.5%. This light VCAM-1 labeling, also noted by immunofluorescence labeling and flow cytometry,[26,28] may be attributed to adherent fragments of VCAM-1$^+$ reticular cells disrupted during the suspension of BM cells.

After in vivo administration of ^{125}I-MoAb PS/2, labeling of low intensity was evidenced by hematopoietic cells of early erythroid, granulocytic, and lymphoid morphology (Fig 6). The radioautographic binding sites were localized to areas of cell surface membrane that often were in contact with either reticular cell processes (Fig 6), or, in outer regions of BM, a prominent electron-dense extracellular matrix (not shown). No MoAb PS/2 labeling was detected on either endothelium or reticular cells.

DISCUSSION

Sustained lymphohematopoiesis in BM cultures requires adhesion between stromal cells and lineage-committed precursors.[5,6] Blocking antibodies have implicated CD44-hyaluronate[32,33] and VCAM-1–VLA-4 in mediating the adhesion.[26-28] The normal in vivo expression of adhesion molecules by BM stromal cells has remained ill-defined, however. Using the in vivo immunolabeling method,[10-14] we have now observed VCAM-1 expression in BM under physiologic circumstances. MAb M/K-2 binding demonstrates that VCAM-1 is constitutively expressed in intact BM in situ, while the labeling seen by EM in normal mice and after γ-irradiation clearly localizes VCAM-1 expression to stromal reticular cells and sinusoid endothelial cells.

The VCAM-1$^+$ reticular cells express VCAM-1 over the entire surface associated with hematopoietic cells of various lineages. The hematopoietic cells themselves express VLA-4, especially at reticular cell contact points. VCAM-1 may, thus, provide a common adhesion molecule by which VLA-4$^+$ cells of various lineages may bind to individual BM reticular cells (Fig 7) capable of providing multiple lineage-specific growth factors.[34] This labeling pattern is in contrast with the expression of another stromal cell determinant, a 100-Kd protein recognized by MoAb KMI-6 and apparently restricted to areas of BM reticular cell surfaces interacting only with lymphoid cells.[35] After γ-irradiation, the KMI-6 determinant is expressed diffusely and in diminished amounts,[35] suggesting that it may be induced by the interacting lymphoid cells. VCAM-1 expression, on the other hand, persists and even apparently increases in intensity when hematopoietic cells have been removed by γ-irradiation. This resembles the increased expression of other adhesion molecules and growth factors reported as a result of ionizing irradiation in a variety of cells.[36,37]

VCAM-1 expression emphasizes the heterogeneity of BM reticular cells in vivo, as in vitro.[5,38,39] The electron-dense cytoplasm and numerous organelles characterizing most VCAM-1$^+$ BM reticular cells suggest much biosynthetic activity. Ionizing irradiation produces a morphologically homogeneous population of reticular cells, all VCAM-1$^+$. It remains to be determined whether their intense labeling by

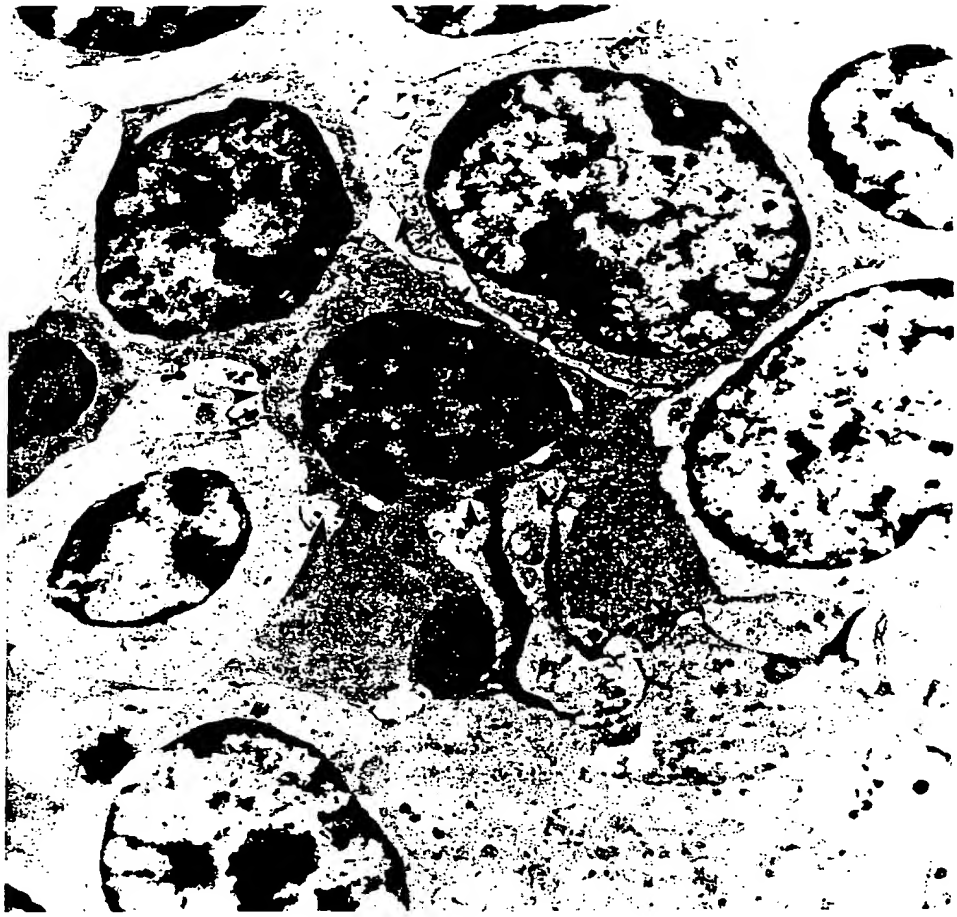

Fig 6. EM radioautograph of a VLA-4$^+$ erythroblast (E) in BM revealed by in vivo binding of ^{125}I-MoAb PS/2. Five radioautographic grains are located over points of contact with reticular cell processes (arrowheads). Radioautographic exposure, 14 weeks; MO × 8,600).

MoAb M/K-2 represents an upregulation of VCAM-1 expression, a selective radiosensitivity of VCAM-1$^-$ stromal cells, or a decreased occupancy of VCAM-1 epitopes by VLA-4.

The VCAM-1 expression by BM reticular cells may serve important functions in vivo. First, it could help lymphohematopoietic cells adhere to the stromal cell network during their differentiation and migration within BM.[12,40] A reduction or conformational change in VLA-4 expression could underlie the release of cells from BM stroma before they enter the blood stream. VCAM-1–mediated adhesion can also facilitate the proliferative action of stromal cell growth factors.[26,28] In addition, it will be interesting to learn if VCAM-1 plays a role in B-cell selection in BM[41] as in germinal centers, where FDC express VCAM-1 constitutively.[42] Germinal center B cells adhere to FDC via VCAM-1,[43] which synergizes with the crosslinking of surface IgM to block apoptosis and promote survival of selected cells.[43] Like germinal center B cells, a large majority of developing B cells in mouse BM normally undergo apoptosis and ingestion by macrophages.[41,44] The possible roles of VCAM-1 or other ligands in this process are unknown. B-lineage cells in BM express, in addition to VLA-4, CD44,[32,33] CD2,[45] and other determinants,[46] while myeloid cells express hemonectin.[47,48] Cell death or survival in BM may well be determined by synergistic combinations of adhesion molecules and receptors.

The integrin VLA-4 anchors cells to fibronectin in extracellular matrix as well as to VCAM-1 on interacting cells.[49] We detect VLA-4$^+$ precursor cells apposed to a thick electron-dense extracellular matrix in subosteal BM,[11,12] where the cells are highly proliferative.[12,40] Lymphohematopoietic precursor cells can bind in vitro to various extracellular matrix components,[50-53] some of which bind cytokines, including granulocyte-macrophage-colony-stimulating factor (GM-CSF), interleukin-3 (IL-3), and IL-7.[54-57] VLA-4 in vivo may, thus, play a role in binding early precursor cells to a subosteal zone of BM extracellular matrix rich in growth factors.

The incidence of VLA-4$^+$ cells now detected radioautographically by MoAb PS/2-binding to BM cell suspensions accords with previous estimates of VLA-4 expression by BM cells.[26,28,58] The lesser degree of labeling revealed in vivo may represent a minimal estimation of VLA-4 expression, with the epitope possibly being partially occupied by VCAM-1 on interacting reticular cells. Indeed, MoAb PS/2 was originally selected by its ability to block adhesion of VLA-4$^+$ cells to stromal cells.[28]

The constitutive expression of VCAM-1 by sinusoid endothelial cells appears to be a unique feature of the BM circulation. In other tissues, vascular endothelium expresses VCAM-1 only when induced by cytokines at sites of inflammation, autoimmune reactions, and neoplasia.[23-25,59,60]

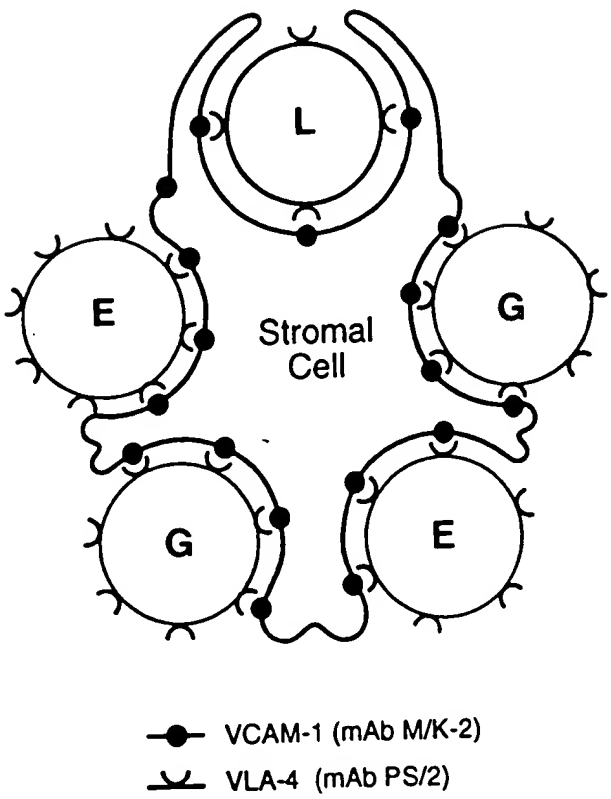

Fig 7. Scheme of VCAM-1 expression by a BM reticular cell interacting with VLA-4$^+$ cells of various hematopoietic lineages. L, lymphoid; G, granulocytic; E, erythroid.

Blood leukocytes rolling along such activated endothelium undergo conformational changes in surface integrins, including VLA-4, strengthening their attachment, before they extravasate between endothelial cells. Anti–VCAM-1 antibodies have been reported not to bind to cultured endothelial cells derived from human BM aspirates.[61] However, our finding that VCAM-1 is constitutively expressed in vivo by endothelial cells throughout the entire sinusoidal pathway in mouse BM suggests that this molecule may be implicated in the cell traffic between BM and blood.

BM is the site of large-scale continuous efflux of locally generated blood cells. Modulation of VLA-4 expression, as well as releasing cells from BM stroma, could be important in allowing cells to pass freely through the VCAM-1$^-$ sinusoid endothelium into the systemic circulation. Newly formed B lymphocytes accumulate within segments of sinusoids, where they resist displacement by perfusion and appear to be adherent to the sinusoidal walls.[10,12,14] The cells may undergo some intravascular maturation before going to the peripheral immune system. This phenomenon, termed "lymphocyte loading," has been attributed to a temporary segmental cessation of sinusoid blood flow.[16] This, however, fails to account for the selective retention of lymphocytes. Our findings raise the possibility that maturing B cells may determine their retention within the lumen by the state of their surface VLA-4, which, for a while, may engage VCAM-1 displayed by the endothelium.

A variety of cells selectively enter BM from the blood. Hematopoietic stem cells injected intravenously as in BM transplantation, rapidly home into recipient BM.[62] Such progenitor cells can also circulate between the various hematopoietic organs. In addition, certain B and T lymphocytes recirculate continuously between blood and BM,[36] while activated memory B cells migrate selectively from spleen to BM, where they form long-lived plasma cells.[17] Although certain lectins have been identified on hematopoietic stem cells,[63] the recognition molecules responsible for BM homing are essentially unknown. As VLA-4 is widely expressed by B and T lymphocytes in the blood, it is possible that binding to BM endothelial VCAM-1, probably in combination with other adhesion molecules, may be involved in the recirculation of cells into BM, thereby differing from recirculation through peripheral lymphoid tissues.[25] The finding of undifferentiated VLA-4$^+$ cells in the sinusoids of γ-irradiated BM raises the possibility that the entry of stem cells into BM may be mediated in part by adhesion to endothelial VCAM-1. A similar mechanism could underlie the entry of neoplastic cells, notably multiple myeloma cells, which express functional VLA-4 and other adhesion molecules.[18] The extensive surface area of BM sinusoidal endothelium and the high rate of blood flow make BM highly efficient in extracting appropriate cells from the blood circulation. Further information on the in vivo expression of adhesion molecules by BM sinusoidal endothelium may suggest means either to enhance the homing of hematopoietic stem cells or to block the entry of neoplastic cells into BM.

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